Establishment of 293 based AAV packaging cell lines using shRNA-mediated *rep* silencing

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Abstract

The main goal of this work was to settle the pillars for establishing stable AAV packaging 293 based cell lines harbouring a mechanism to control Rep induced cytotoxicity, based on shRNA-mediated silencing and the Cre/loxP system.

Four different *rep*-silencing shRNAs were successfully designed and cloned separately between loxP sites. In their presence, *rep* expression should be silenced. Infection with an adenovirus expressing Cre (AdV-Cre) will excise the shRNA-coding sequence reconstituting *rep* expression.

The system was tested in transient and stable expression and in the latter challenged through AdV infection. In transient, *rep* mRNA levels showed to be similar in all transfected cells, regardless of shRNA presence. In the presence of *rep*, *cap* expression was 5-fold higher than *rep.* The selected stable populations had integrated copies of packaging plasmid components ranging from 0 to 1 and *cap* expression was lower, when compared to transient. After AdV-Cre infection of the stable control population harbouring *rep* and *cap* without shRNA, we observed an increase in *rep-cap* levels. However, in the *rep*-silenced populations, *rep* mRNA levels remained low before and after infection. Thus, it was not possible to validate the silencing effect of each shRNA. In this work we conclude that either multiple copies or high expressing locus should be pursued to express *rep*. Overall this work contributed to establish important methods and generated knowledge that will be used to establish high producer AAV cell lines, namely through the further improvement of the design, selection and delivery of the shRNAs and *rep-cap* expression cassettes.

1 Introduction

Adeno-associated virus (AAV) is a small virus with a single-stranded DNA genome of 4.7 kilobases, flanked by 145 base pairs inverted terminal repeats (ITRs). The genome comprises three promoters (p5, p19 and p40) and two genes (*rep* and *cap*). p5 and p19 control the *rep* gene, which encodes for four Replication proteins (Rep78, 68, 50 and 42) while p40 activates the transcription of the *cap* gene, originating the capsid proteins (VP1, VP2 and VP3) [1]. AAV is considered replication-defective since it needs the presence of a helper virus, such as Adenovirus (AdV) or Herpes Simplex Virus (HSV) to replicate [1]. AAV has been widely used as a recombinant delivery system for gene therapy since it is non-pathogenic [2], presents low cytotoxicity [3], transduces dividing and non-dividing cells [4], has different serotypes which allow tissue tropism [5] and has been documented to present long-term transgene expression in humans [6]. Recombinant AAV (rAAV) consists on the transgene expression cassette flanked by AAV ITRs. *rep* and *cap* sequences as well as helper virus functions are provided in *trans* [7].

One of the major drawbacks for the application of rAAV in a clinical setting is the establishment of cost-effective and scalable manufacturing systems that provide high titers [8]. Stable cell lines can be easily characterized, allow for scale-up and produce relatively high vector titers with

increased reproducibility and for more prolonged periods [9]. This type of cell lines has been developed from HeLa, A549 and 293 cells. The latter constitutively express adenoviral genes *E1a/b* which allows the use of non-pathogenic replication defective AdV, adding an extra safety level. However, it has been shown that the *E1* gene triggers the production of AAV Rep proteins, which leads to cytostatic and cytotoxic effects [14], [15]. Hence, *rep* gene expression needs precise regulation.

The main goal of this work is to establish the pillar methodologies to develop stable AAV packaging 293 based cell lines harbouring a control mechanism for viral *rep* gene expression. This system is based on RNA interference, namely short hairpin RNA – shRNA, specific for *rep* silencing and Cre recombinase activity. Thus, four shRNAs (1-4) were originated that specifically target different parts of the *rep* gene. In the presence of a *rep*-specific shRNA (flanked by loxP sites), AAV Rep expression is expected to be silenced. Then, upon infection with a non-replicative AdV-Cre, the loxP-shRNA site should be excised, restoring *rep* expression.

2 Materials and methods

shRNA design and ssDNA annealing. The design of four shRNA sequences targeting the AAV2 *rep* gene (shRNA1 – 4) and the non-target scrambled control (shRNAscr) was based on results delivered by three different bioinformatics tools, namely BLOCK-iT™ RNAi Designer (Thermo Fisher Scientific, Maryland, USA), GPP Web Portal [16] (Broad Institute; Massachusetts, USA) and GenScript siRNA Target Finder [17] (GenScript, New Jersey, USA). Complementary single-stranded oligonucleotides were synthesized (IDT, Iowa, USA) and then annealed to generate the double-stranded oligonucleotides, which was conducted for 4 minutes at 95ºC followed by 25 minutes at 25ºC.

Construction of *rep*-specific shRNA expressing constructs. Plasmid backbone pRC contains i) the AAV2 *rep-cap* genes (Genbank NC_001401.2, nt 190 – 4469), ii) wt *loxP* sequences flanking human U6 promoter and *Sbf*I/*Mlu*I restriction sites (U6 as in pLKO.1, Sigma-Aldrich, Missouri, USA), iii) a SV40 polyA iv) a chicken β-globin HS4 core sequence (Addgene #44352 [18]) and v) a hPGK-GFP-P2A-BSD fusion gene ([19], [20] and Addgene #12252, kindly provided by Dr. Didier Trono). To construct the shRNA-coding plasmids (pRC-sh1-4 and pRC-shscr), 60 ng of the double-stranded oligonucleotides coding for each shRNA were used for cloning into pRC, double digested with *Sbf*I and *Mlu*I. Control plasmid pΔRepC was generated through the digestion of pRC with *Nru*I and *Swa*I to remove the *rep* gene and then circularization using T4 ligase. Plasmid sequence was confirmed using restriction endonucleases and by sequencing using SUPREMERUN service (Eurofins Genomics, Val Fleuri, Luxembourg).

Cells lines and culture conditions. 293 (ATCC, American Type Culture Collection, CRL-1573) is a human embryonic kidney cell line containing Adenovirus 5 *E1a/b* genes. This cell line was used for transient transfections and stable population establishment. Cells were grown in Dulbecco's modified Eagle's Medium (DMEM; Gibco™ – Thermo Fisher Scientific, Massachusetts, USA) supplemented with 10% (v/v) Fetal Bovine Serum (FBS; Gibco™ – Thermo Fisher Scientific, Massachusetts, USA), under adherent conditions using standard polystyrene treated cell culture flasks

(Corning Life Sciences, New York, USA). All cells were cultured at 37 ºC in a humidified atmosphere with 8% (v/v) CO_2.

Transfection of 293 cells with polyethylenimine (PEI). 293 cells were seeded at $1x10^5$ cells/cm^2, to reach 70-80% confluency at time of transfection (24 hours post-seeding). For transient transfection, PEI (Polysciences Inc., Hirschberg, Germany) was used in a total of 3 µg DNA/10^6 cells in a 1:1.5 (w/w) DNA:PEI ratio. However, for traceability and quality control purposes, when transfecting cells for the development of stable populations, linear 25 kDa PEIpro® (Polyplus transfection, Illkirsch, France) was used in a 1:1 (w/w) DNA:PEI ratio with 5 µg DNA/10^6 cells. The culture medium was exchanged 4 to 6 hours post-transfection (hpt). Cells were analysed 48 hpt by fluorescence microscopy, using a Leica DMI6000 inverted microscope (Olympus), and flow cytometry using BD FACSCelesta™ (BD Biosciences, California, USA). For the establishment of stable packaging populations, 48 hpt, cells were seeded into selection medium: DMEM 10% (v/v) supplemented with Blasticidin (InvivoGen, Toulouse, France). Cells in selection were observed every 48 hours and if necessary, they were passaged, re-splitted and/or new selection medium was added. During the selection procedure, cells were analysed by flow cytometry to evaluate GFP percentage.

qPCR analysis. 5 µL of each sample was added to a 20 µL final volume qPCR reaction with LightCycler® 480 Probes Master Mix (Roche Life Science, Penzberg, Germany) and appropriate primers/probe sets at a final concentration of 0.5 µM and 0.25 µM, respectively. Thermal cycling was carried out in a LightCycler® 480 Real Time PCR System (Roche Applied Science, Penzberg, Germany), at 95°C for 10 minutes, 45 cycles of 95°C for 10 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 1 second (single acquisition) and a cooling step at 40 °C during 10 seconds. For all analyses, technical replicates with standard deviation (SD) higher than 0.3 were not considered. For integrated copy number calculation, total cellular DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. 150 ng of genomic DNA was used per PCR reaction. Copy numbers were calculated by normalizing to the human albumin gene (ALB), with an assumed number of 2 copies per 293 cell. For the relative gene expression assays, RNA was extracted using QIAamp® RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was synthesized using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Penzberg, Germany), following manufacturer's instruction. Gene expression was normalized to two reference genes (RG - UBC and TOP1) using the $\Delta\Delta C_T$ method [21].

3 Results

3.1 Establishment of stable AAV2 *rep*-specific shRNA packaging populations

The AAV packaging populations based on 293 cells were established through the transfection with plasmids pRC, pΔRepC, pRC-sh1-4 and pRC-scr, originating populations RepCap, ΔRep, RC-sh1–4, RC-shscr, respectively using 5 µg DNA/10^6 cells and PEIpro in a 1:1 DNA:PEI ratio. 48 hpt all transfected cells presented a remarkable impact in cell survival and morphology **(Figure 3.1A)**. Since it was also observed in the ΔRep population, unable to express the *rep* gene, it might indicate a phenomenon of PEIpro-induced cytoxicity. Flow cytometry analysis revealed different transfection

efficiencies, ranging from 8% to 27% (**Figure 3.1B**) being the highest in cells harbouring the plasmid without the *rep* gene (ΔRep) and the lowest in cells transfected with the scrambled control (RC-shscr). Cells were selected in the presence of blasticidin for almost nine weeks, until populations presented around 90% of GFP positive cells. RepCap took the longest time to recover while the rest of the populations were selected almost at the same pace. After selection, all populations presented the same growth and proliferation profile as parental 293 cells, determined by cell confluency observation.

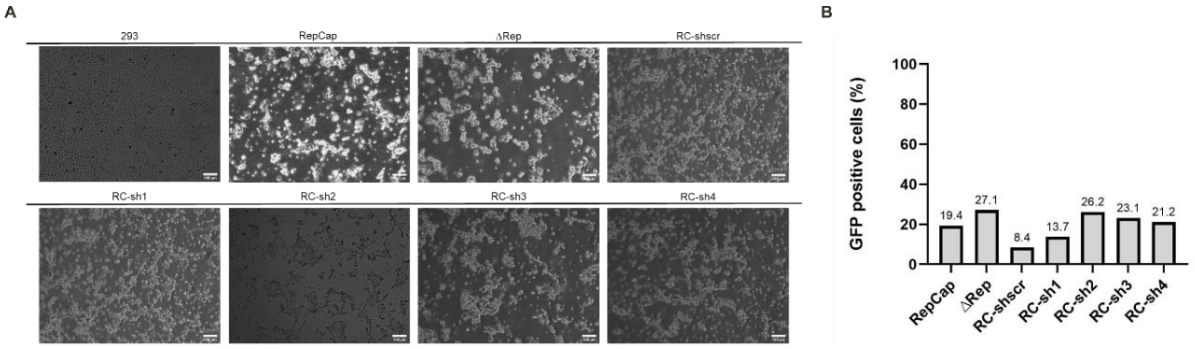

Figure 3.1: Evaluation of cell confluency and transfection efficiency for the establishment of AAV2 *rep*-specific shRNA packaging populations. 293 cells were transfected with different plasmids at **5** µg/10^6 cells at a DNA:PEIpro ratio of 1:1 (w/w) and collected 48 hpt for analysis by **(A)** phase contrast microscopy and **(B)** flow cytometry (n=1). The scale bar corresponds to 100 µm.

3.2 Transient transfection of AAV2 *rep*-specific shRNA packaging plasmids

A transfection protocol optimization with pRC was performed, identifying 3 µg DNA/10^6 cells as the condition that delivered the best transfection efficiency with reduced cell death (data not shown). Then, 293 cells were transfected with the packaging plasmids with this condition, using in-house PEI in a DNA:PEI ratio of 1:1.5. Transfection efficiencies remained below 30% (**Figure 3.2B**) and cell death was still present in almost every transfection, except for cells transfected with pRC-sh4 and scr, which also have the lowest transfection efficiency (**Figure 3.2B**). When comparing phase contrast microscopy images of cells transfected in this assay (**Figure 3.2A**) with the images of the establishment of stable populations (**Figure 3.1A**), cells have an overall better morphology in the first one, which means that DNA ratio alongside type of PEI used in the population establishment might be causing increased cytotoxicity.

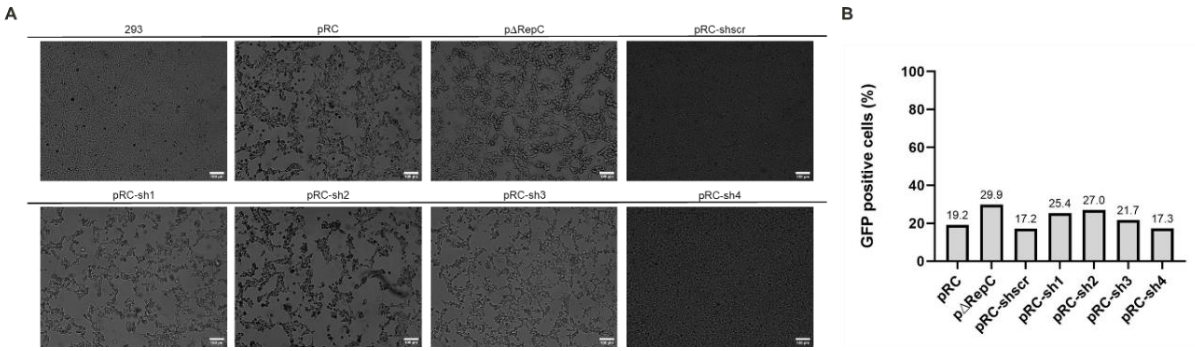

Figure 3.2: Evaluation of cell confluency and transfection efficiency in cells transiently transfected with the AAV2 *rep*-specific shRNA packaging plasmids. 293 cells were transfected with different plasmids at **3** µg/10^6 cells at DNA:PEI ratio of 1:1.5 (w/w) and collected 48 hpt for analysis by **(A)** phase contrast microscopy and **(B)** flow cytometry (n=1). The scale bar corresponds to 100 µm.

3.3 Gene expression analysis

Expression of AAV genes as well as *GFP* was analysed 48 hpt and in the established stable populations by RT-qPCR. As depicted in **Figure 3.3A**, *rep*, *cap* and *GFP* were expressed 48 hpt and as expected, at higher levels than in stable populations (**Figure 3.3C**). No *rep* gene silencing potential was observed with the four designed shRNAs in transiently transfected cells (pRC-sh1 – 4) since their *rep* levels were similar to pRC transfection (**Figure 3.3B**).

In stable, *rep* mRNA levels in populations RC-sh1 and RC-sh3 were slightly higher (below 2-fold) compared to RepCap population (**Figure 3.3D**). *rep* expression was not detected in RC-sh2 and RC-sh4 and RC-shscr (**Fig. 3.3C**).

Regarding *cap* mRNA levels in transient, they were 2.5 to 7.2-fold higher than *rep* levels (**Figure 3.3A**). In the absence of *rep* (pΔRepC), the levels of *cap* mRNA were the lowest (**Figure 3.3A**), being 6-fold inferior when compared to pRC (**Figure 3.3B**). Stable packaging populations show a decreased level of *cap*, which seems not related with *rep* levels (**Figure 3.3C**).

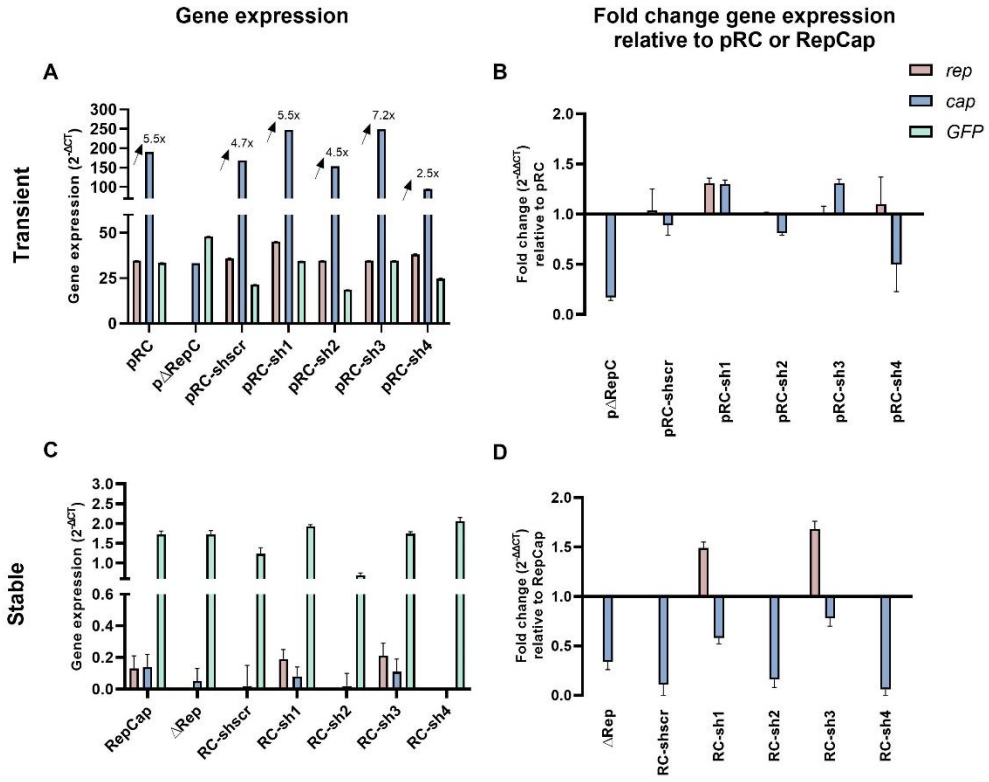

Figure 3.3: Relative gene expression analysis of *rep*, *cap* and *GFP* in (A and B) transiently transfected cells (48 hpt) and (C, D) stable populations. cDNA was synthesized from 2 µg of total RNA extracted. Gene expression was assessed by RT-qPCR normalized against TOP1 and UBC reference genes within the same sample and are shown as gene expression ($2^{-\Delta CT}$) **(A and C)** or fold change relative to the respective levels in control cells harbouring the pRC plasmid ($2^{-\Delta\Delta CT}$) **(B and D)**. **(A)** Arrows indicate the **cap* expression ratio vs *rep* expression in the same sample. Data is shown by mean ± standard deviation (A and B n= 1; C and D n= 3).

3.4 Integrated plasmid copy number

As shown in **Table 3.1**, the integrated plasmid copy number varied between 0 and 1 in all cell populations, for all tested genes. Furthermore, the populations which do not express *rep* (RC-sh2, 4 and scr – **Figure 3.3C**) do not have any *rep* integrated copies, which can explain the lack of

expression. There are some inconsistencies in amplified copy number (**Table 3.1**) and mRNA levels (**Figure 3.3C**) in RC-sh1 detecting 0 *rep* copies and a gene expression of 0.19, and populations RC-shscr, 2, 3 and 4 detecting 0 copies of *GFP* but having a gene expression of 1.23, 0,69, 1.74 and 2.06.

Table 3.1: Analysis of integrated plasmid copy number. qPCR analysis of *rep*, *cap* and *GFP* plasmid sequences was carried out using primers/probe specific sets. Copy number calculation was performed through the normalization to ALB gene copies, with an assumed number of 2. The data is shown by mean ± standard deviation (n=3).

	Copy number		
Cell population	***rep***	***cap***	***GFP***
293	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.00
RepCap	1.00 ± 0.12	1.00 ± 0.30	1.00 ± 0.32
ΔRep	0.00 ± 0.02	0.00 ± 0.10	1.00 ± 0.19
RC-shscr	0.00 ± 0.00	0.00 ± 0.02	0.00 ± 0.11
RC-sh1	0.00 ± 0.26	1.00 ± 0.30	1.00 ± 0.30
RC-sh2	0.00 ± 0.00	1.00 ± 0.19	0.00 ± 0.26
RC-sh3	1.00 ± 0.10	1.00 ± 0.07	0.00 ± 0.12
RC-sh4	0.00 ± 0.04	0.00 ± 0.09	0.00 ± 0.29

3.5 Characterization of AAV2 *rep*-specific shRNA packaging cell populations after AdV-Cre infection

To assess the effects of helper virus factors in the amplification of AAV viral genes and *GFP*, RepCap, ΔRep, RC-sh1 and RC-sh3 stable populations were infected with AdV-Cre and AdV-Control, at a MOI of 5. 24 hours post-infection, all infected populations exhibited cytopathic effects typical of adenoviral infection: loss of cell adhesion and cell rounding (data not shown). 42 hours post-infection, *rep*, *cap* and *GFP* levels were analysed by qPCR and RT-qPCR. Overall, both adenovirus infections did not increase DNA copy number, apart for the RepCap population, that increased from 1 to 2 *rep* copies when infected with AdV-Control (**Table 3.2**). It is important to point out that AdV altered RG expression (data not shown) so normalization to the non-infected controls could not be performed. Regarding *rep* expression, it was only slightly amplified in RepCap population, upon AdV infection (below 2-fold) (**Figure 3.4A**), which is coherent with *rep* DNA amplification regarding AdV-Control infection (**Table 3.2**). Furthermore, there is no *rep* expression amplification of RC-sh1 and 3, relative to RepCap after AdV-Cre infection (**Figure 3.5B**). *cap* gene expression appeared to have increased very slightly with levels below 0.1 in population RC-sh1 and increased around 3-fold and 0.5-fold in populations RepCap and RC-sh3, respectively, upon AdV infection (**Figure 3.4B**). Still, *cap* mRNA amplification was not accompanied by *cap* DNA increase (**Table 3.2**). As expected, *cap* levels were the lowest in the population deleted for the *rep* gene (ΔRep) (**Figure 3.4B**). Again, *rep* and *cap* copy number of RC-sh1 (~0) are not coherent with mRNA levels shown in all tested conditions (**Figure 3.4A** and **B**). To confirm gene expression tendencies, more biological replicates need to be performed.

Table 3.2: Analysis of plasmid copy number amplification before and after AdV infection. qPCR analysis of *rep*, *cap* and *GFP* plasmid sequences was carried out using primers/probe specific sets. Copy number calculation was performed through the normalization to Albumin gene copies, with an assumed number of 2 (n=1). The data is shown by mean ± standard deviation of technical replicates (n=1).

	***rep* copy number**			***cap* copy number**		
Cell population	**No infection**	**AdV-Control**	**AdV-Cre**	**No infection**	**AdV-Control**	**AdV-Cre**
293	0.00 ± 0.03	0.00 ± 0.08	0.00 ± 0.05	0.00 ± 0.01	0.00 ± 0.03	0.00 ± 0.05
RepCap	1.00 ± 0.03	**2.00 ± 0.02**	1.00 ± 0.01	1.00 ± 0.06	1.00 ± 0.04	1.00 ± 0.06
ΔRep	0.00 ± 0.10	0.00 ± 0.06	0.00 ± 0.01	0.00 ± 0.03	0.00 ± 0.14	0.00 ± 0.13
RC-sh1	0.00 ± 0.04	0.00 ± 0.03	0.00 ± 0.07	0.00 ± 0.02	0.00 ± 0.10	0.00 ± 0.14
RC-sh3	1.00 ± 0.02	1.00 ± 0.06	1.00 ± 0.19	1.00 ± 0.02	1.00 ± 0.06	1.00 ± 0.03

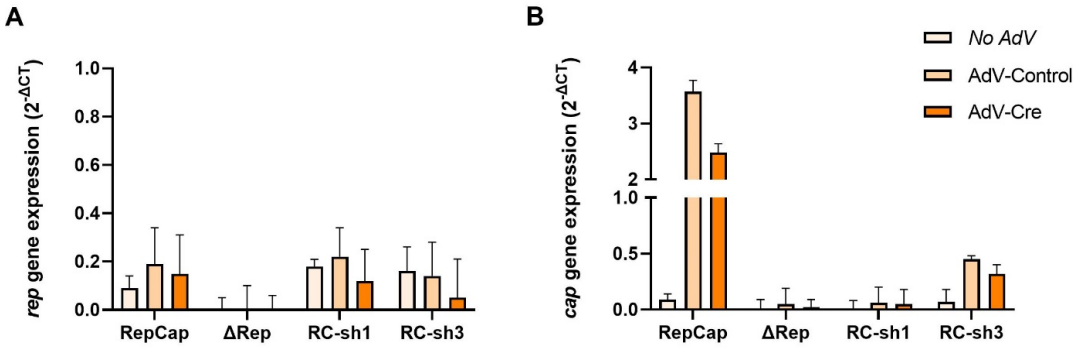

Figure 3.4: Gene expression analysis of *rep* (A) and *cap* (B) of RepCap, ΔRep, RC-sh1 and RC-sh3 stable populations, non-infected and infected with AdV-Control and AdV-Cre (42 hours post-infection). cDNA was synthesized from 2 µg of total RNA extracted. Gene expression was assessed by RT-qPCR normalized against TOP1 and UBC reference genes within the same sample and are shown as gene expression ($2^{-\Delta CT}$). Data is shown by mean ± standard deviation of technical replicates (n=1).

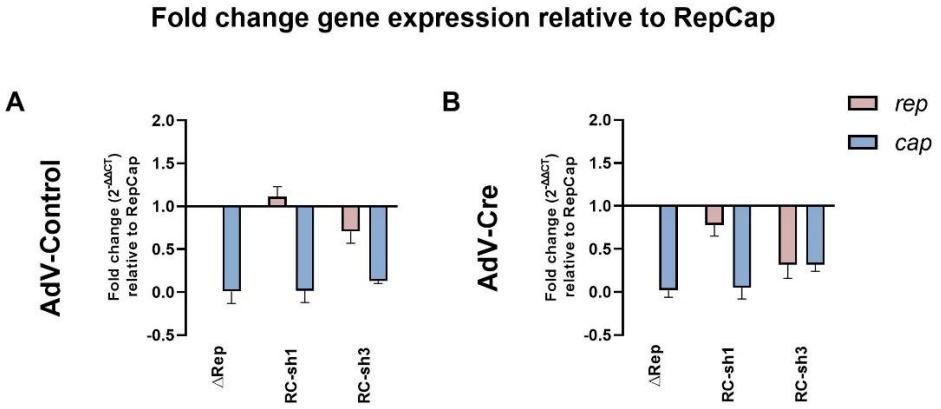

Figure 3.5: Fold change of *rep* and *cap* gene expression relative to RepCap population of stable populations ΔRep, RC-sh1 and RC-sh3 infected with AdV-Control (A) and AdV-Cre (B) (42 hours post-infection). cDNA was synthesized from 2 µg of total RNA extracted. Gene expression was assessed by RT-qPCR normalized against TOP1 and UBC reference genes within the same sample and are shown as fold change relative to the respective levels in control cells harbouring the pRC plasmid - RepCap ($2^{-\Delta\Delta CT}$). Data is shown by mean ± standard deviation of technical replicates (n=1).

4 Discussion and conclusions

Stable cell line generation through clone selection and characterization involves a significant time and effort. So, for the sake of time, population establishment was performed first, with 5 µg/10^6

cells of each generated plasmid using a certified PEI (PEIpro, Polyplus). 48 hpt, a decreased cell survival was observed in all transfected cells, including the ones that received the vector deleted for the *rep* gene (pΔRepC) (**Figure 3.1A**). Transfection efficiencies were variable, and the highest percentage was achieved with plasmid pΔRepC and the lowest with pRC-shscr (**Figure 3.1B**). We can hypothesize that the observed cytotoxicity can be derived from the expression of the *rep* gene, the amount of delivered DNA and/or the type of transfection reagent. When transfecting cells with a previously optimized condition of 3 µg/10^6 cells using in-house PEI, transfection efficiencies were within expected (**Figures 3.2B**). If we compare phase contrast microscopy images of stable populations establishment (**Figure 3.1A**) and this assay (**Figure 3.1B**), cell morphology was better in the latter, which indicates that in fact, PEI-pro and DNA quantity have an impact in cell survival and morphology.

Gene expression results indicate that none of the used shRNAs were able to knockdown the *rep* gene. In transient, analysis of *rep* gene expression, relative to control (pRC), revealed similar levels of *rep* mRNA in all transfected cells (**Figures 3.3A and B)**. However, some reports point that shRNA-mediated gene silencing can be effective 48 hpt [22], [23]. When the *rep* gene is expressed, the levels of *cap* expression were 2.5 to 7.2-fold higher than *rep* (**Figure 3.3A**). Comparatively, in cells transfected with pΔRepC, levels of *cap* mRNA where 6-fold reduced compared to transfection with pRC (**Figures 3.3A and B**). The expression of the Rep proteins is probably acting as a positive regulator of the p40 promoter, leading to an increased expression of *cap* mRNA, an observation in line with published results [24]. Regarding GFP expression, it seems to be higher in cells deleted for the *rep* gene (pΔRepC) (**Figure 3.3A**). Accordingly, it has been documented that Rep is able to downregulate expression from heterologous promoters [25].

To confirm the shRNA properties as well as to characterize the selected populations, *rep-cap* gene expression and amplification were quantified by qPCR, before and after AdV infection. In the absence of helper factors, *rep* expression was not detected in RC-sh2, RC-sh4 and RC-shscr populations (**Figure 3.3C**) while RC-sh1 and RC-sh3 had a slight increase compared to control, RepCap (below 2-fold) (**Figures 3.3D**). To investigate whether the absent/low expression of *rep* (and consequently *cap*) could be due to a downregulation driven by shRNA, the integrated plasmid copy number of *rep*, *cap* and *GFP* was also assessed. At the beginning of work, it was hypothesized that if the shRNAs were efficient in silencing *rep* expression, then clones with higher integrated plasmid copy number could have endured. Populations RepCap and RC-sh3 have an average number of 1 copy of *rep* gene while RC-sh1 did not reach 1 (**Table 3.1**). Populations RC-sh2, 4 and RC-shscr do not have any *rep* integrated copies, which can explain the lack of *rep* gene expression (**Table 3.1** and **Figure 3.3C**). For other genes, the range was also between 0 and 1 (**Table 3.1**). It is important to point out that since these samples are populations, there is cell heterogeneity, meaning that the copy numbers obtained reflect only an average and do not represent each individual cell. Additionally, since these cells were transfected with a circular plasmid, that can be randomly linearized within the cell, there is an increased probability of generating clones that do not integrate essential genes. As such, it has been shown that the number of stable colonies can be increased through vector linearization [26]. It can also be hypothesized that despite the presence of Rep proteins, the lack of AAV ITRs in the

constructions might have had a negative impact on integration. Some studies show that there is higher integration efficacy when using ITRs [27] while others report that these structures are not required for integration and do not affect integration efficacy [28]. Additionally, the severe cytotoxicity upon transfection during the establishment of stable populations may have compromised the survival of clones with a higher integrated plasmid copy number.

Stable cell lines tend to mimic post-adenoviral infection wt AAV life cycle through the amplification of *rep* and *cap* genes [13], which is related to successful rAAV production [10]–[13]. Thus, RepCap, ΔRep, RC-sh1 and RC-sh3 were infected with AdV-Cre and AdV-Control. For RepCap population, a slight increase in *rep* is observed (below 2-fold), after AdV infection (**Figure 3.4A**). For other tested populations, there was no *rep* expression amplification after AdV-Cre infection, when compared to RepCap (**Figure 3.5B)**, which further reinforces the hypothesis that shRNA1 and 3 do not repress *rep*. Contrarily of what was expected, especially for the RC-sh3 population, *cap* expression seems to have been amplified after AdV infection (**Figures 3.4B**). *cap* gene expression is in fact amplified during AdV infection, however, it relies on Rep78-mediated transactivation of the p40 promoter [29], [30]. In the future, a relative quantification with a standard curve should be employed to understand if this reported result is real or an artefact originated due to the normalization of abnormally expressed RG (data not shown). Regarding plasmid integrated copy number in stable cell lines, some reports attribute no clear relation with rAAV productivity [10], while others still consider that possibility [12]. After infection, only RepCap population showed *rep* DNA amplification from 1 to 2 copies, upon AdV-Control (**Table 3.2**), which aligns with the slight increase in *rep* mRNA levels, and consequently *cap* expression (**Figure 3.4A and B**). The combined results of this work seem to point that only 1 copy of *rep-cap* per cell is too low to trigger the desirable levels of *rep-cap* amplification required for an increased rAAV production. In line with our results, Chadeuf *et al.* (2000) [31] developed a 293 based AAV stable cell line with 1-2 *rep* integrated copies which ended up yielding 27-fold less AAV vectors than control 293 cells.

Although, no AAV2 packaging cell line harbouring a *rep*-silencing mechanism based on shRNA was established, this work gave useful insights regarding transfection and *rep*-mediated cytoxicity, shRNA design and delivery, which will shape future assays. Moreover, the implementation and validation of several qPCR methods are valuable for upcoming wok. The development of a screening method for high producer clones, which relates integrated copy number, gene expression and/or protein production with rAAV productivity, as preliminarily explored in this work, is of great interest and should be further investigated.

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